

2016

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Allen, R.

Allen, R. (2016) 'The cytotoxic and genotoxic potential of titanium dioxide (TiO<sub>2</sub>) nanoparticles on human SH-SY5Y neuronal cells in vitro', The Plymouth Student Scientist, 9(2), p. 5-28.

<http://hdl.handle.net/10026.1/14126>

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The Plymouth Student Scientist

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# **The cytotoxic and genotoxic potential of titanium dioxide (TiO<sub>2</sub>) nanoparticles on human SH-SY5Y neuronal cells *in vitro***

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## **Abstract**

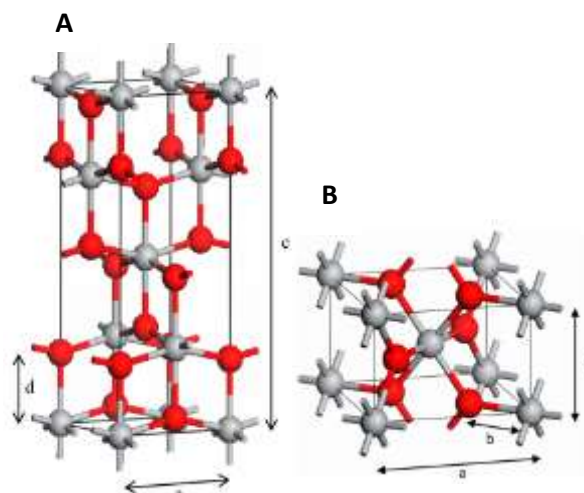
Titanium dioxide (TiO<sub>2</sub>) nanoparticles are one of the most commonly used nanomaterials. They are used in plastics, sunscreens, personal care products, pharmaceuticals and the food industry due to their photocatalytic properties, high refractive index and generally unreactive nature. Their vast range of applications, and hence potential exposure to humans, raises concern over the safety of the nanomaterial. TiO<sub>2</sub> nanoparticles have been extensively studied; however, their toxic effect on humans is still poorly understood, particularly the adverse effects they may have on the nervous system. *In vivo* studies have shown that TiO<sub>2</sub> nanoparticles are able to enter the brain via circulation and through the olfactory pathway. Because of their potential genotoxicity and carcinogenic effect, the aim of this study was to assess the cytotoxic and genotoxic potential of TiO<sub>2</sub> nanoparticles on human SH-SY5Y neuronal cells. Anatase TiO<sub>2</sub> nanoparticles were used in this experiment due to their extensive use in industry. Results obtained from this study show that TiO<sub>2</sub> nanoparticles are able to induce DNA damage in neuronal cells at concentrations of 3000 μM (239.61 μg/ml) after 24 and 48 hours of exposure, but do not significantly alter cell viability even at concentrations as high as 10,000 μM (798.66 μg/ml). Cell morphology, as determined by scanning electron microscopy, was also unaltered in this study. Further study is required to determine cell internalisation and the mechanisms behind the genotoxicity induced by TiO<sub>2</sub> nanoparticles.

## Introduction

Nanotechnology is the manipulation and engineering of materials with at least one dimension  $<100\text{nm}$  in length (Oberdörster *et al.*, 2007). In recent years the demand for engineered and manufactured nanomaterials has increased rapidly (Borm and Berube, 2008) due to their vast range of applications in medicine, the food industry and energy conversion (Seigrist *et al.*, 2008). Current applications of nanomaterials include; anti-microbial food processing and packaging, antibacterial milk bottles for babies (Siegrist *et al.*, 2008), and water purification (Liga *et al.*, 2011). Due to their small size and large surface area to volume ratio, nanoparticles are readily transported around the body, able to enter cells and capable of releasing drugs over time enabling the production of nano scale implants and drug delivery systems (Langer and Weissleder, 2015; Roco, 2003).

Titanium Dioxide ( $\text{TiO}_2$ ) is one of the most commonly used nanoparticles due to its photocatalytic properties, high refractive index (Borm and Berube, 2008), thermal stability and its generally insoluble, unreactive nature (Lee *et al.*, 1985). It is used as a pigment in antifouling paints (Borm and Berube, 2008), plastics, enamels, foods, sunscreens, pharmaceuticals, personal care products such as toothpaste, shampoos and deodorants (Weir *et al.*, 2012) and in the treatment of drinking water (Liga *et al.*, 2011). The vast use of  $\text{TiO}_2$  nanoparticles in everyday products and foods has sparked a great interest in the safety of the nanomaterial, particularly after the International Agency for the Research of Cancer (IARC) have classified pigment grade  $\text{TiO}_2$  as a potential carcinogen to humans (Wang *et al.*, 2008a). Nanomaterials have different physiochemical properties to their bulk forms, such as size, surface area:volume, surface charge, redox activity and surface hydrophobicity, and therefore have different mechanisms of toxicity. As a result, not only do nanomaterials provide a benefit to industry but also a risk (Oberdörster *et al.*, 2007; D'agata *et al.*, 2013).

The toxicity of  $\text{TiO}_2$  has been studied in a variety of conditions to assess its safety in humans and in the environment. Previous studies have reported  $\text{TiO}_2$  induced cytotoxic effects such as altered cell cycle, shrinkage of nuclear membranes and apoptosis (Acar *et al.*, 2015; Coccini *et al.*, 2015; Gosch *et al.*, 2010; Hu *et al.*, 2011; Valdiglesias *et al.*, 2013; Wang *et al.*, 2007), inhalation risks and correlation with Crohn's disease in humans (Weir *et al.*, 2012), as well as gill pathologies and biochemical brain disturbances in fish (Boyle *et al.*, 2013; Federici *et al.*, 2007; Ramsden *et al.*, 2009). Studies have also found that  $\text{TiO}_2$  nanoparticles are capable of causing DNA damage (Jugan *et al.*, 2012; Petkovic *et al.*, 2011; Shukla *et al.*, 2011; Trouiller *et al.*, 2009; Valdiglesias *et al.*, 2012; Vevers and Jha, 2008), which is increased with the interaction of UV light (Vever and Jha, 2008) due to its photocatalytic properties (Borm and Berube 2008).  $\text{TiO}_2$  is considered a polymorphic material as it is found in three different forms; Rutile, Anatase and less commonly, Brookite. The property of  $\text{TiO}_2$  and hence its toxicity, is closely related to its crystal structure. Anatase and Rutile are the two forms of  $\text{TiO}_2$  used most. Anatase has more applications in industry than the other two crystal forms due to its high specific surface area and increased activity in photocatalysis (Bourikas *et al.*, 2014; Li *et al.*, 2004), it is however considered to be far more toxic than rutile (Weir *et al.*, 2012).



**Figure 1.** Comparative tetragonal structures of (A) anatase and (B) rutile crystal forms of  $\text{TiO}_2$ . Grey balls represent titanium and red balls represent oxygen (adapted from Bourikas *et al.*, 2014).

Previous studies have shown that the crystalline structure and size of  $\text{TiO}_2$  determines the toxicity, aggregation and agglomeration of the nanoparticle. Smaller particles of  $\text{TiO}_2$  have been shown to induce higher levels of inflammation in the lung when inhaled (Oberdörster *et al.*, 2007) and are migrated around the body more readily than bulk  $\text{TiO}_2$  (Ma *et al.*, 2010). In comparison to this D'agata *et al.*, (2013) found that although nano-sized  $\text{TiO}_2$  showed higher levels of accumulation, bulk  $\text{TiO}_2$  actually caused a higher genotoxic effect in marine mussels. Similarly Magdelenova *et al.*, (2011) found that dispersions with larger agglomerates of  $\text{TiO}_2$  NPs induced greater cell damage than dispersions with smaller agglomerates. Published studies on the toxicity of  $\text{TiO}_2$  are conflicting, reflecting the great effect aggregation, agglomeration, size of nanoparticle and crystal structure can have on the property of  $\text{TiO}_2$  (Li *et al.*, 2004; Ma *et al.*, 2010; Oberdörster *et al.*, 2007).

Humans are exposed to  $\text{TiO}_2$  nanoparticles via multiple exposure routes on a daily basis. One route of exposure is through the consumption of food and water, either directly, as a result of packaging or through environmental pollution and bioaccumulation within the food chain. Previous research has assessed the content of  $\text{TiO}_2$  in food and water. Gottschalk *et al.*, (2009) found there to be  $0.021\mu\text{g/L}$   $\text{TiO}_2$  present in surface water and  $4.28\mu\text{g/L}$  present in waste water treatment plants. Foods with particularly high values of  $\text{TiO}_2$  NPs include coffee, sweets and chewing gum. It is estimated that the average 3-4 year old male in the western world will consume  $2\text{mg TiO}_2/\text{kg BW}$  per day, (Weir *et al.*, 2012).

Once in the body, nanoparticles can enter the bloodstream and be taken up by cells (Rothen-Rutishauser *et al.*, 2006), effecting vital organs such as the liver, brain and placenta (Borm and Berube, 2008).  $\text{TiO}_2$  NPs can affect the brains of offspring, inducing brain damage when present in the abdominal cavity (Ma *et al.*, 2010) and effecting behaviour and reproduction rates in future generations (Jacobasch *et al.*, 2014). Previous work has shown that nanoparticles form nanomaterial-protein

coronas when in the blood stream as plasma proteins are adsorbed to the surface of the nanoparticle, conferring their biological identity and masking the nanoparticle, enhancing cell uptake (Cedervall *et al.*, 2007; Doak *et al.*, 2009; Lynch and Dawson, 2006). Apolipoprotein E, a lipoprotein involved in brain trafficking (Lynch and Dawson, 2006), has been found to associate with some nanoparticles, facilitating transport to the brain and across the blood brain barrier (BBB) (Bramini *et al.*, 2014; Kim *et al.*, 2007; Michaelis *et al.*, 2006). Although a lot of nanoparticles are more likely to accumulate in lysosomes, some nanoparticles are small enough to cross the blood brain barrier (BBB) and accumulate in regions of the brain such as the olfactory bulb and the hippocampus (Borm and Berube, 2008; Bramini *et al.*, 2014; Hu *et al.*, 2011). The mechanisms for the uptake of the NPs across the BBB appears to be receptor – mediated endocytosis in endothelial cells in the brain (Brun *et al.*, 2012; Wohlfart *et al.*, 2012). Czajka *et al.* (2015) illustrates the potential routes of exposure and mechanisms of TiO<sub>2</sub> nanoparticle induced toxicity on the nervous system.

Humans are also exposed to nanoparticles through the air. Manufactured and engineered nanoparticles could potentially erode to form nano scale particulate matter, exposing humans to NPs in the air (Handy and Shaw, 2007). The vast majority of research on inhaled TiO<sub>2</sub> nanoparticles has been carried out on lung cells however; inhaled TiO<sub>2</sub> can also reach the brain via the olfactory pathway (Elder *et al.*, 2006; Wang *et al.*, 2008a; Wang *et al.*, 2008b). Once inhaled TiO<sub>2</sub> can accumulate in areas such as the olfactory nerve layer, olfactory ventricle, cerebral cortex, thalamus, and hippocampus regions CA1 and CA3 (Wang *et al.*, 2008a).

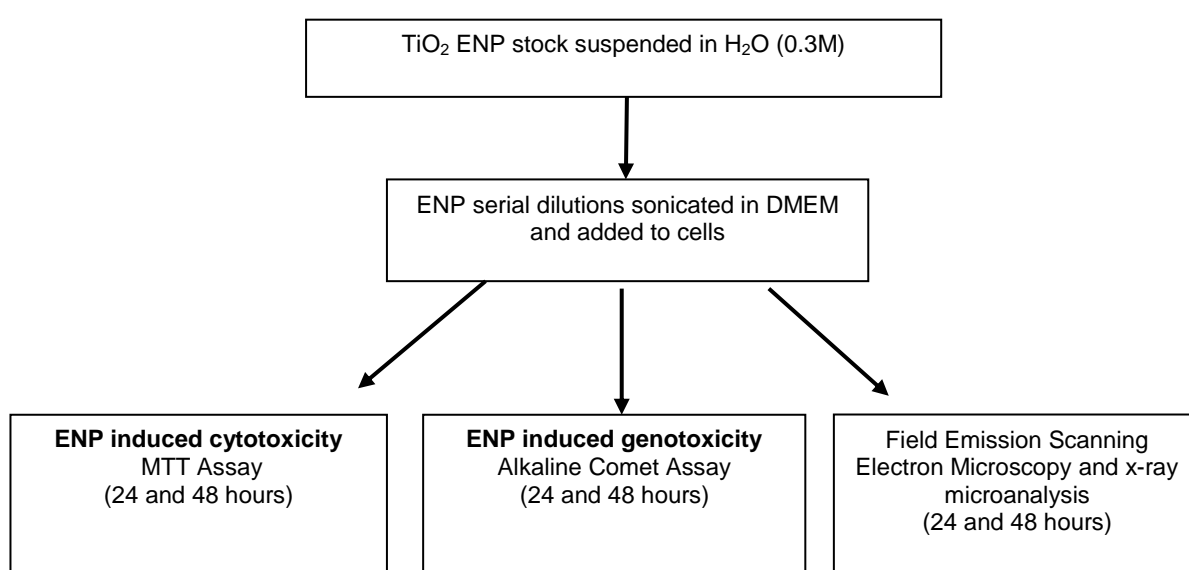
Once in the brain TiO<sub>2</sub> nanoparticles mainly accumulate in the hippocampus causing lipid peroxidation, protein oxidation, oxidative damage (Wang *et al.*, 2008a; Wang *et al.*, 2008b), a decreased antioxidant capacity and an increase in the production of reactive oxygen species (ROS) (Hu *et al.*, 2011). Other effects in the brain include inhibited proliferation, induced morphological changes, apoptosis (Márquez – Ramirez *et al.*, 2012), shrinkage of nuclear membranes (Hu *et al.*, 2011), changes in copper (Cu), potassium (K) and Zinc (Zn) levels (Federici *et al.*, 2007) and dysregulation of the BBB (Brun *et al.*, 2012).

Given the potential carcinogenic effect of TiO<sub>2</sub> nanoparticles, the aim of this study was to assess the cytotoxic and genotoxic potential of TiO<sub>2</sub> nanoparticles on the developing nervous system using undifferentiated SH-SY5Y cells. SH-SY5Y cells are undifferentiated noradrenergic cells capable of exhibiting dopamine  $\beta$  hydroxylase, cloned from a four year old female with neuroblastoma. The neuroblastoma cells are sub cloned from cell line SK-N-SH, and are used largely in neuroscience (Filograna *et al.*, 2015). Cell viability was determined using the methyl thiazole tetrazolium (MTT) assay and DNA damage was assessed using the alkaline comet assay. Scanning electron microscopy was used to assess morphology of the cells and x-ray microanalysis was used to analyse the presence, aggregation and location of the nanoparticles. In order to validate these assays copper was used as a reference toxic agent.

## Materials and Methods

### Chemicals

Anatase TiO<sub>2</sub> (nanopowder, <25 nm particle size, 99.7% trace metals basis, CAS: 1317-70-0, Batch # 07324KD) and low melting point agarose gel (LMPA) (CAS: 39346-81-1) were purchased from Sigma Aldrich. Cupric Sulphate Pentahydrate (CuSO<sub>4</sub>H<sub>2</sub>O) ACS grade (CAS: 7758-99-8) was purchased from BDH chemicals LTD. MTT (Thiazolyl blue tetrazolium bromide, C<sub>18</sub>H<sub>16</sub>N<sub>5</sub><sup>SBr</sup>, CAS: 298-93-1) was purchased from Melford Chemical and Biochemical Manufacturing. Dulbecco's modified Eagle's medium (DMEM, Gibco) was purchased through Thermo Fisher Scientific. Sterile A Dulbecco's Phosphate Buffered Saline (DPBS) and Sterile A 0.25% trypsin were purchased from Gibco by Life Technologies.



**Figure 2.** Schematic diagram for TiO<sub>2</sub> ENP toxicity assessment methods.

### Cell Culture

Human neuroblastoma SH-SY5Y cells were purchased from ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA, USA) supplemented with 10% foetal bovine serum (FBS) and 0.5% Penicillin Streptomycin. Cells were maintained in a humidified incubator with 5% CO<sub>2</sub> and 95% air at 37°C in filtered cap flasks (CELLSTAR). To passage cells, all media was removed and cells were washed with 4 ml Dulbecco's Phosphate buffered saline before adding 500µl 0.25% trypsin, 0.53mM EDTA to detach cells with 3.5ml fresh DMEM. To ensure the cells had detached from the flask, cells were viewed under an inverted light microscope. Cell counts were carried out using a haemocytometer.

### TiO<sub>2</sub> Nanoparticle Preparation

Anatase TiO<sub>2</sub> NPs were suspended in sterile distilled water at a stock concentration of 0.3M, and sonicated using a Fisher Scientific sonicator. Serial dilutions were made to obtain concentrations ranging from 10µM to 10,000µM (0.79866 µg / ml – 798.66 µg / ml). TiO<sub>2</sub> 0.3M stock was sonicated for 1 hour 30 minutes prior to making diluted concentrations to ensure nanoparticles were suspended and to prevent aggregation

and agglomeration. Individual concentrations were mixed with a pipette and sonicated for at least 5 minutes prior to cell exposure. All concentrations of TiO<sub>2</sub> had equal amounts of PBS to water and TiO<sub>2</sub> to ensure ratio of water to DMEM media was not influencing cell health. Controls were used with equal amounts of media, PBS and water/TiO<sub>2</sub> suspension, made up of 8:1:1 parts respectively.

### **Validation of Assays with Copper**

Prior to experimentation, MTT assays were carried out in SH-SY5Y cells exposed to Cupric Sulphate Pentahydrate (CuSO<sub>4</sub>5H<sub>2</sub>O) for 24 and 48 hours to obtain a concentration of copper suitable for use as a positive control throughout experiments in order to validate each assay. The concentration of copper within the Cupric Sulphate Pentahydrate was calculated. CuSO<sub>4</sub>5H<sub>2</sub>O was then used as a positive control throughout all 24 and 48 hour MTT experiments, SEM analysis and Comet assays at a copper concentration of 300µM.

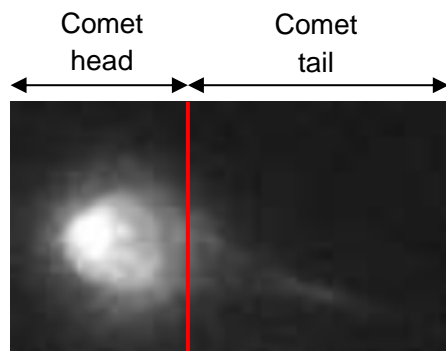
### **MTT assay**

Cell viability was assessed using the methyl thiazole tetrazolium (MTT) assay. The assay assesses the capability of viable cells to transform the methyl thiazole tetrazolium into a formazan dye. Cells were seeded at a density  $1 \times 10^4$  /ml onto a 96 well plate, 100µl/well. MTT was prepared in DPBS 5mg/ml. After exposure periods of 24 and 48 hours, all media was removed from wells and replaced with 85µl DMEM and 15µl MTT and incubated for 3 hours at 37°C. Blue formazan crystals inside cells were dissolved by adding 100µl Dimethyl sulfoxide (DMSO) to each well and incubating for a further 10 minutes at 37°C. Contents of wells were mixed thoroughly before the absorbance was read at 595nm using a VERSA max microplate reader. Media blanks were used to eliminate the interference of the DMEM media and MTT with the results. TiO<sub>2</sub> blanks, comprising of media, TiO<sub>2</sub> NP suspension and MTT were used to prevent the interference of the nanoparticles with the absorbance obtained. Blank values were subtracted from concentration values and represented as a percentage of the control. All experiments were carried out in triplicate (three wells per plate) and repeated four times.

### **Assessment of DNA damage by alkaline comet assay (Single-cell gel electrophoresis)**

Cells were seeded in a 6 well plate at a density of  $1 \times 10^4$  / ml exposed to titanium dioxide nanoparticles for periods of 24 and 48 hours. Cells were exposed to concentrations of 30 µM, 300 µM and 3000 µM TiO<sub>2</sub> NPs. Cells were trypsinized and centrifuged. Cell suspension was mixed with low melting point agarose gel and pipetted onto normal melting point agarose (NMPA) coated slides, and covered with cover slips. Slides were refrigerated at 4°C for an hour. Cover slips were removed and slides were placed back to back in lysis buffer and refrigerated at 4°C for a further hour. Slides were then placed in electrophoresis chamber and left to unwind for 20 minutes. Electrophoresis chamber ran for 20 minutes at 25V. Slides were then placed in a coplin jar with neutralisation buffer for 10 minutes. Slides were left to air dry for scoring. Slides were stained with 20µl of ethidium bromide (20 µg /ml) and scored using a fluorescent microscope and Comet IV software. 50 cells per gel were scored, with duplicate slides per treatment condition and exposure period. Copper was used as a positive control during this experiment at both 24 and 48 hour exposure periods. %Tail DNA was used to measure genotoxicity caused by TiO<sub>2</sub> as it is a recommended parameter to analyse from the alkaline comet assay for

regulatory purposes as it has the best correlation with dose. % tail DNA consists of DNA released as fragments and relaxed loops (Kumaravel and Jha, 2006).



**Figure 3.** Image of comet head and tail taken from SHSY5Y cell exposed to 300 $\mu$ M TiO<sub>2</sub> nanoparticles for 48 hours following fluorescent staining (as seen in figure 11).

Figure 3 shows a typical 'comet', a cell with a visible 'tail' of DNA. Images of comets are portrayed in each figure for their respective concentrations. A longer tail and corresponding increased % tail DNA indicates an increase in DNA strand breaks. Tails of comets are hard to identify in all images obtained from these results but are slightly more apparent at higher concentrations.

### SEM Analysis

Field emission scanning electron microscopy (Field Emission SEM; JEOL JSM-7001F) was used to observe any morphological differences. Cells were grown on plastic cover slips (Melinex) in a twelve well plate, seeded at a density of  $1 \times 10^4$ /ml. Cells were exposed to concentrations of 300 $\mu$ M and 1000 $\mu$ M TiO<sub>2</sub> nanoparticles for 24 and 48 hours before fixation. All media and TiO<sub>2</sub> concentrations were removed and cells were fixed in their plate using 2.5% glutaraldehyde (pH 7.2) and 0.1M Sodium cacodylate buffer for one hour. Glutaraldehyde was removed and cover slips containing cells were placed into individual containers with fresh 0.1M Sodium cacodylate buffer. Cells were dehydrated with a series of ethanol concentrations ranging from 10% to 100%, with 15 minute intervals between each concentration. Ethanol was replaced with liquid carbon dioxide and dehydrated using a critical point dryer (Emitech K850 Critical Point Dryer). Cells were then mounted on stubs and coated in carbon using a Quorum Q150 T-ES Carbon Coating Unit. Copper was used as a positive control to compare cell morphology to.

### X-ray microanalysis

X-ray microanalysis was carried out on control cells, and cells exposed to 300 $\mu$ M and 1000 $\mu$ M TiO<sub>2</sub> nanoparticles for 24 and 48 hours. Cells were coated in carbon due to its low atomic number, allowing easier identification of other elements with a high atomic number. Copper was used as a positive control for x-ray microanalysis. Element maps and spectrums were produced when elements with a high atomic number were identified using back scattered electron microscopy.



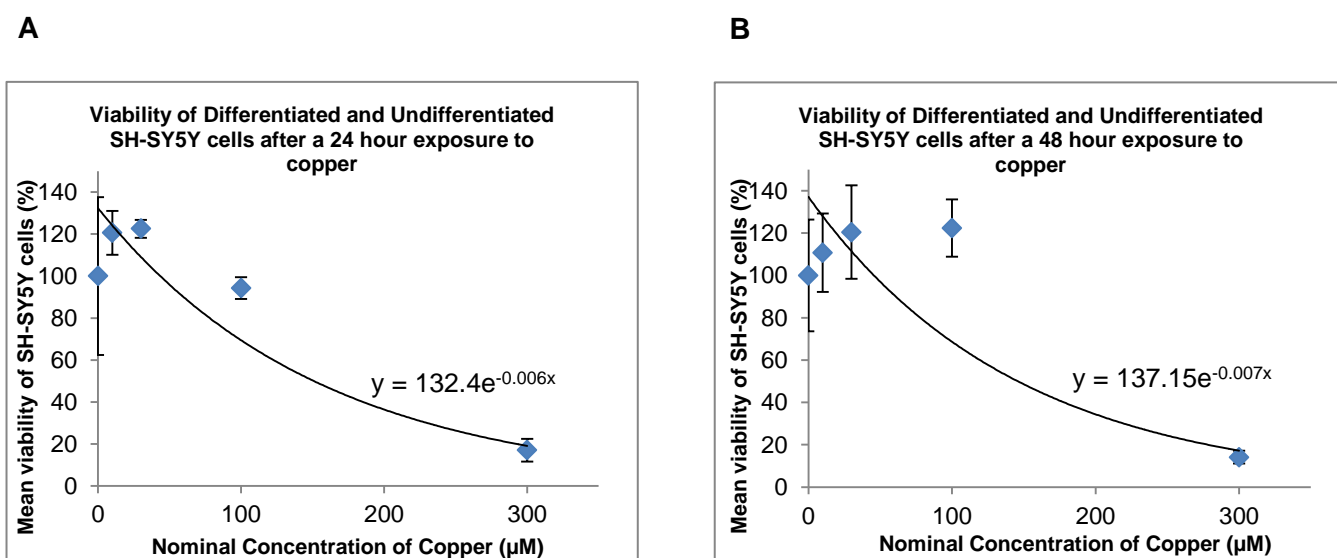
## Statistical analysis

In order to determine if all data was parametric, normal distribution was tested using the Shapiro-Wilk test and equality of variance was tested using Levene's test. Data from MTT was normally distributed and had an equality of variance ( $P > 0.05$ ) so a one way analysis of variance (ANOVA) with an LSD post hoc test was used at a significance level of  $P < 0.05$ . Data for comet assay was not normally distributed and variances are unequal ( $P < 0.05$ ) so a Kruskal-Wallis H test was used to determine if there was a significant difference between results. Mann-Whitney U tests were used between all concentrations and controls used in the comet assay ( $P < 0.05$ ). All statistics were carried out in SPSS.

## Results

### Validation of MTT Assay

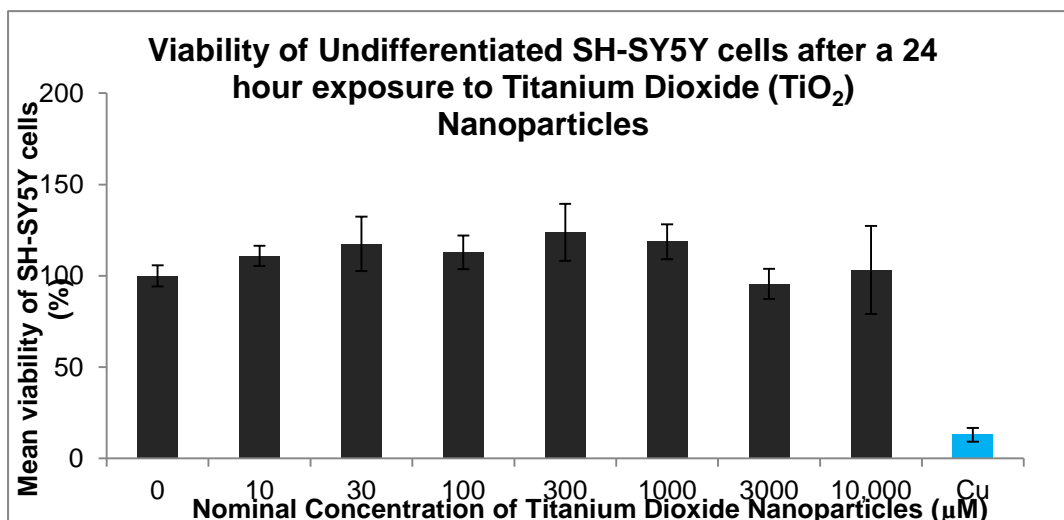
Cells were exposed to Copper Sulphate Pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) for exposure periods of 24 and 48 hours in order to obtain a concentration of copper suitable for use as a positive control for experiments. At copper concentrations of  $300\mu\text{M}$  a significant decrease in cell viability was observed.  $300\mu\text{M}$  copper was used as a positive control in all other assays throughout the project.



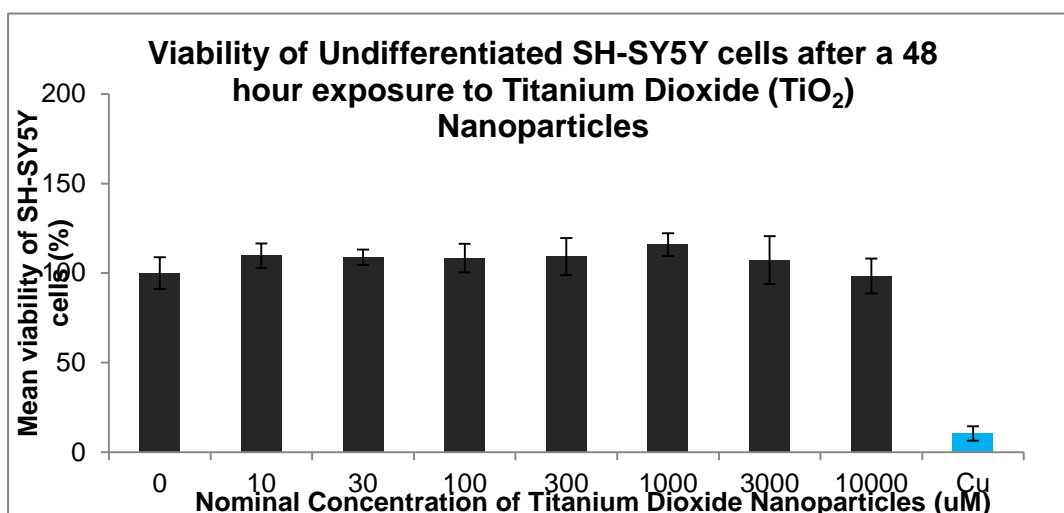
**Figure 4.** Viability of differentiated and undifferentiated SH-SY5Y cells after a 24 hour (A) and 48 hour (B) exposure to Copper Sulphate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) represented by means  $\pm$  SD and line of best fit.

### Cell Viability

As seen in figures 5 and 6, no significant decrease ( $P > 0.05$ ) in cell viability was observed in response to any of the concentrations of  $\text{TiO}_2$  nanoparticles compared to the control following exposure periods of 24 (figure 6) and 48 hours (figure 6). Copper was used as a positive control at  $300\mu\text{M}$  in all repeats of these experiments.



**Figure 5.** Cell Viability of differentiated and undifferentiated SH-SY5Y cells following a 24 hour exposure to TiO<sub>2</sub> nanoparticles presented by mean  $\pm$  SD (N=3) with positive control (300µM Cu).

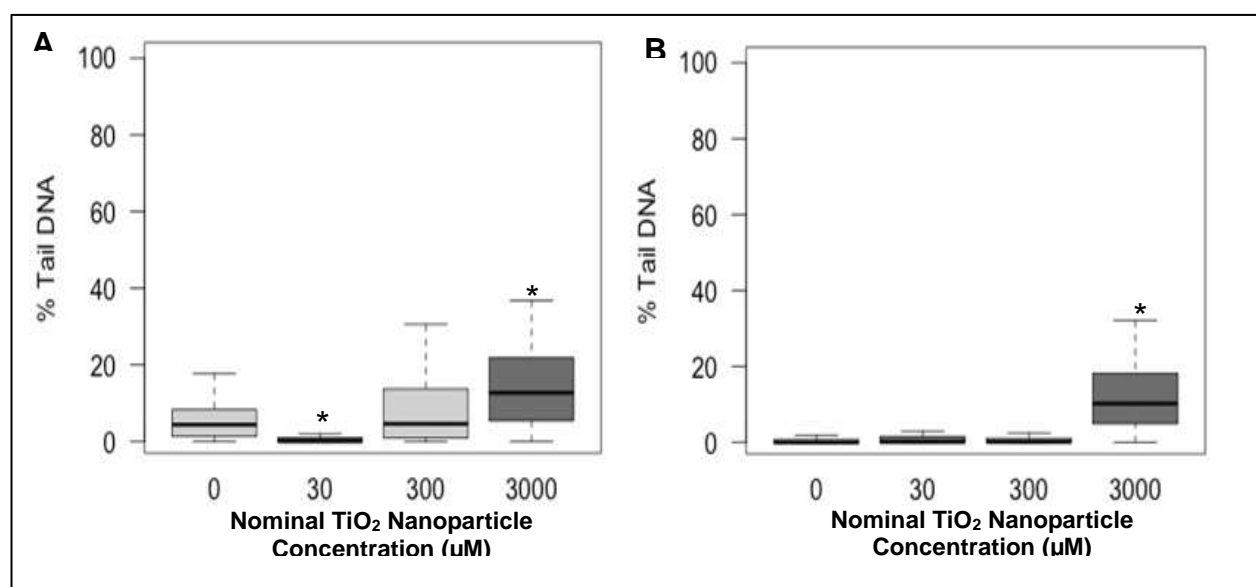


**Figure 6.** Cell viability of differentiated and undifferentiated SH-SY5Y cells following a 48 hour exposure to TiO<sub>2</sub> nanoparticles presented by means  $\pm$  SD (N=3) with positive control (300µM Cu).

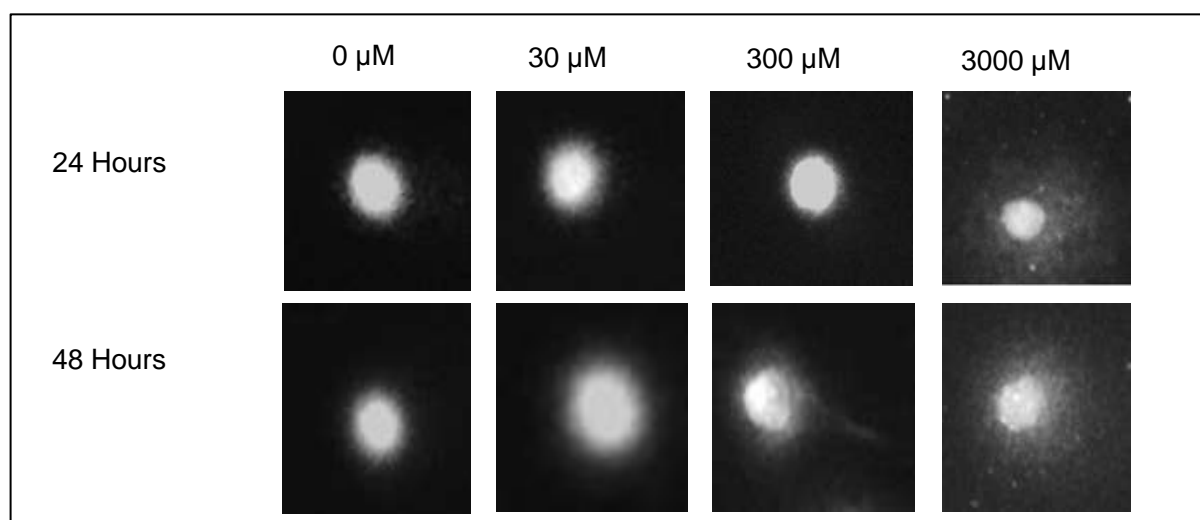
### Assessment of DNA damage using the Alkaline Comet Assay (Single gel electrophoresis)

An alkaline comet assay (single gel electrophoresis) was carried out to assess the genotoxic potential of TiO<sub>2</sub> nanoparticles at concentrations of 30 µM, 300 µM and 3000 µM. Kruskal Wallis H tests revealed significant differences in % tail DNA following exposure periods of 24 and 48 hours, ( $P < 0.01$ ). Mann Whitney U tests were then performed between concentrations. A significant difference was observed

between the control and 3000  $\mu\text{M}$  following both 24 and 48 hours of exposure with increased % tail DNA ( $P < 0.01$ ) as seen in figure 8.  $\text{TiO}_2$  nanoparticles at the highest concentration used (3000  $\mu\text{M}$ ) were able to induce DNA damage following exposure periods of 24 and 48 hours. A significantly lower % tail DNA was observed following 24 hours of exposure to 30  $\mu\text{M}$   $\text{TiO}_2$  nanoparticles, assessed by the Mann-Whitney U test ( $P < 0.01$ ). Although statistically significant, further tests need to be conducted to improve experimental validity. Copper was used as a positive control in this study, however due to loss of cell viability; cells were not present for scoring purposes.



**Figure 7.** Box and whisker plots for comet assay values (% Tail DNA) following a 24 hour (A) and 48 hour (B) exposure to  $\text{TiO}_2$  nanoparticles. Boxes plots show 25<sup>th</sup> and 75<sup>th</sup> percentile values, with median value and 90<sup>th</sup> percentile values (whiskers). The asterisk (\*) represents a significant ( $P < 0.01$ ) difference in % tail DNA compared to the control.

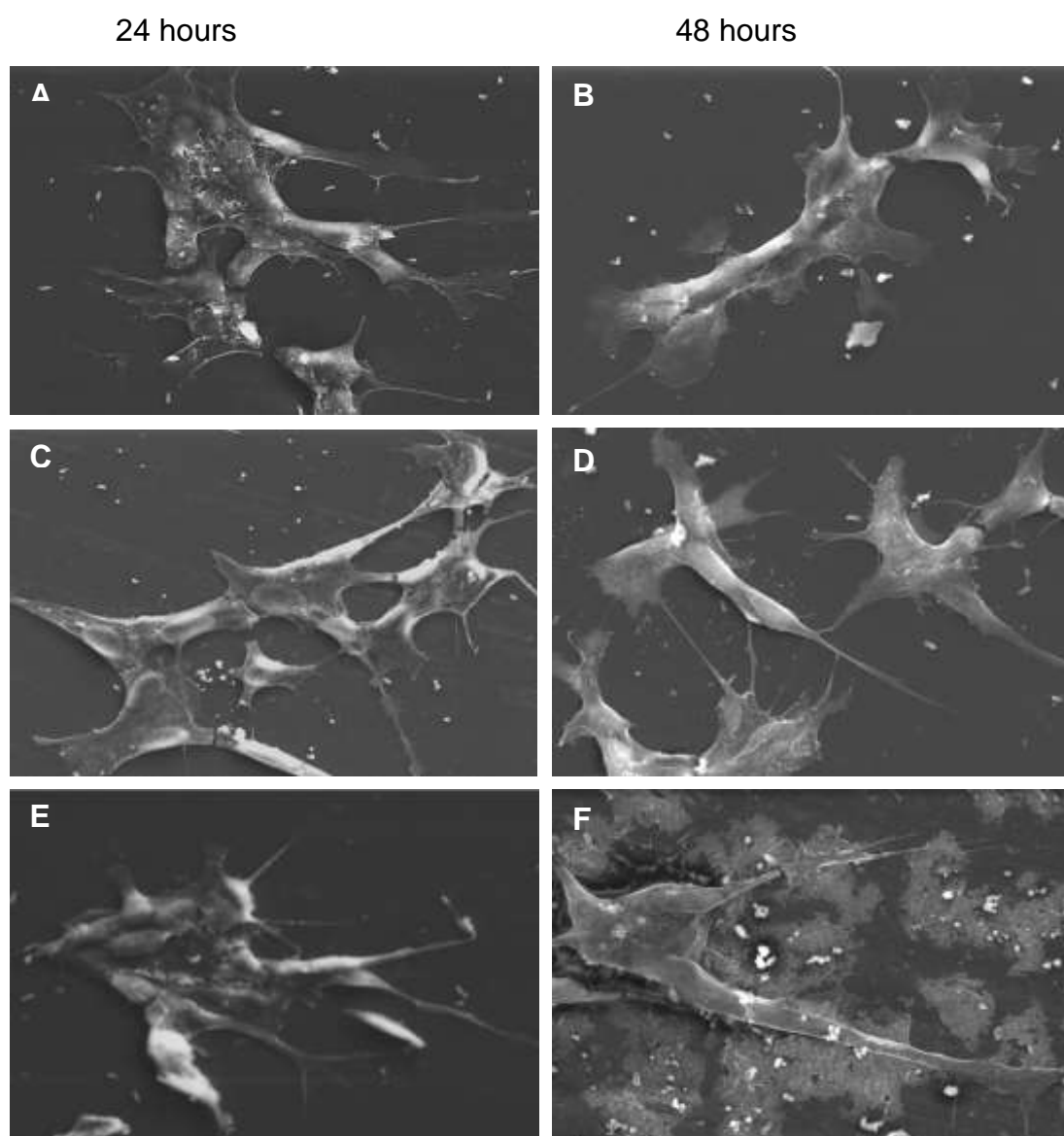


**Figure 8.** DNA comets following single gel electrophoresis after 24 and 48 hour exposure periods to varied concentrations of  $\text{TiO}_2$  nanoparticles (30  $\mu\text{M}$ , 300  $\mu\text{M}$  and 3000  $\mu\text{M}$ ).

Figure 8 shows DNA comets from each treatment condition. A clear difference can be seen between the cells in the control group and those exposed to 3000  $\mu\text{M}$   $\text{TiO}_2$  nanoparticles, with an increased portion of migrated DNA outside of the nucleus (tail DNA) in the 3000  $\mu\text{M}$  treated cells. No DNA comets were present for copper treated cells due to loss of cell viability.

### **Morphology of cells**

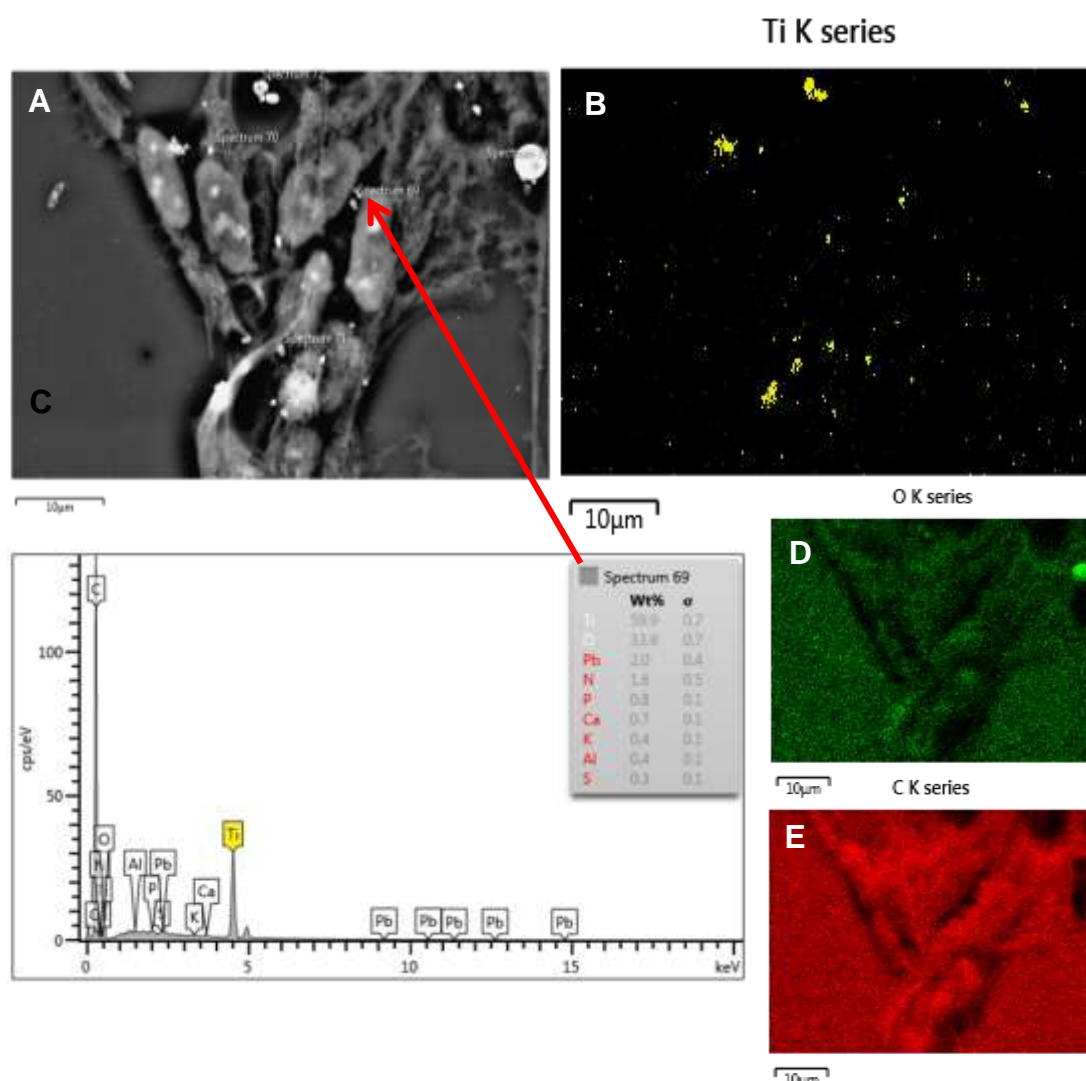
No clear differences in cell morphology was observed between control cells and cells exposed to  $\text{TiO}_2$  nanoparticle concentrations of 300 $\mu\text{M}$  and 1000 $\mu\text{M}$ . Cells were also exposed to copper to provide a positive control for the experiment however, cell morphology could not be assessed due to excessive cell lysis and detachment from the melinex cover slip. Damage can be seen in all images potentially caused from dehydration and fixation.



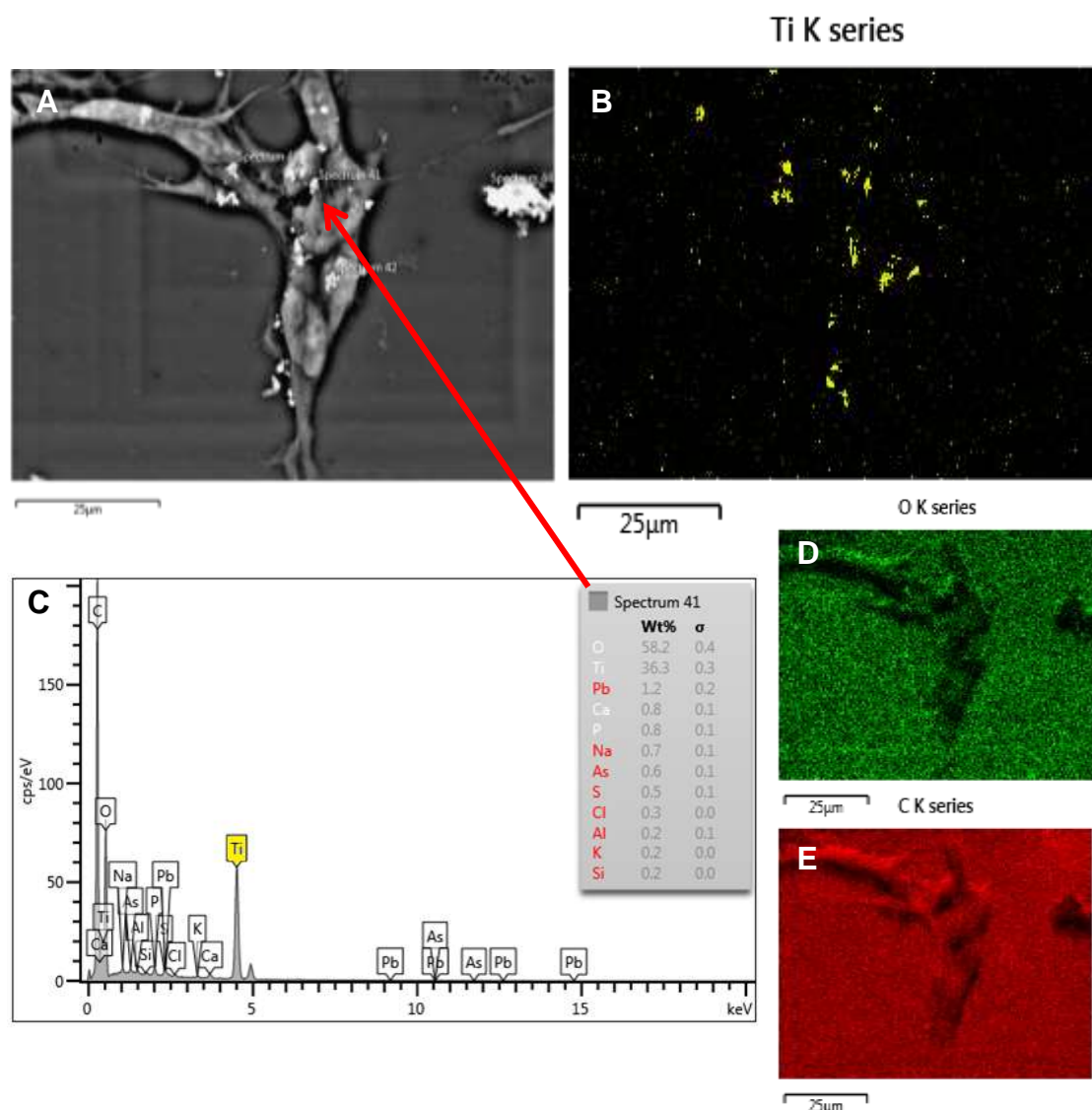
**Figure 9.** Morphology of control cells (A and B), cells treated with 300 $\mu\text{M}$   $\text{TiO}_2$  nanoparticles (C and D) and cells treated with 1000 $\mu\text{M}$   $\text{TiO}_2$  nanoparticles (E and F) for exposure periods of either 24 hours (A, C and E) or 48 hours (B, D and F).

# X-ray microanalysis

Back – scattered electron microscopy was used to detect the presence of heavy elements with a high atomic number in the sample and further SEM/EDS analysis was carried out to produce maps and spectrums of elements present. Samples were carbon coated for spectrum analysis causing the carbon peaks seen in figures 11B and 12B. Titanium (11E and 12E, represented in yellow) was found in aggregates attached to the surface of the cells at both concentrations of 300µM and 1000µM, but not in control samples (data not shown) following 24 and 48 hours of exposure. Titanium was also found in the media at both concentrations of 300µM and 1000µM, but was more commonly observed attached to the surface of the neuroblastoma cells. X-ray microanalysis could not be carried out on cells exposed to copper due to excessive cell death and detachment from the coverslip.



**Figure 10.** (A) SH-SY5Y cell following 48 hour exposure to 300µM (23.9598 µg / ml) TiO<sub>2</sub> nanoparticles. (C) EDS spectrum showing peaks of C (E), O (D), Ti (B), Pb, Ca, P, Na, As, S, Cl, Al, K and Si at location 69 as shown by red arrow.



**Figure 11.** (A) SH-SY5Y cell following 48 hour exposure to 1000 $\mu$ M (79.866  $\mu$ g / ml) TiO<sub>2</sub> nanoparticles. (C) EDS spectrum showing peaks of C (E), O (D), Ti (B), Pb, Ca, P, Na, As, S, Cl, Al, K and Si at location 41 as shown by red arrow.

## Discussion

Despite extensive *in vitro* and *in vivo* research into the safety of Titanium dioxide nanoparticles, their mechanisms and levels of toxicity are still poorly understood, particularly in the nervous system, with conflicting and variable results throughout the literature. This study assessed the cytotoxic and genotoxic potential of TiO<sub>2</sub> nanoparticles on the developing nervous system using undifferentiated SH-SY5Y cells. The cells are derived from human brain tumours, however are often used in neurotoxicity assessments due to their dopaminergic properties and biochemical characteristics similar to neurons (Filograna *et al.*, 2015).

This study found that TiO<sub>2</sub> nanoparticles, even at concentrations much higher than environmentally relevant (3000 $\mu$ M and 10,000 $\mu$ M), had no impact on mitochondrial function, as determined by MTT assay, with no significant difference of cell viability

between treated cells and the control ( $P > 0.05$ ). Despite no significant difference in cell viability it can be seen in figures 6 and 7 that 300 $\mu$ M and 1000 $\mu$ M respectively, have an absorbance higher than that of the control. This indicates a potential interference of TiO<sub>2</sub> with the assay. Nanomaterials are known to interact with colorimetric and fluorometric dyes used in assays such as the MTT, often giving false results for cytotoxicity, (Doak *et al.*, 2009; Wörle-Knirsch *et al.*, 2006). Blanks containing TiO<sub>2</sub> were used in this assay to account for changes in absorbance caused by TiO<sub>2</sub> nanoparticles, however it is unclear how much TiO<sub>2</sub> was present in the wells containing the SH-SY5Y cells. As seen in figures 10 and 11, the nanoparticles can be seen adhered to the cells rather than dispersed in the media. If the TiO<sub>2</sub> has a greater tendency to attach to the cells than remain dispersed in the media, then TiO<sub>2</sub> will remain in wells even after media is removed causing an increased absorbance respective to the blanks. It is understood that different cytotoxicity assays can give different results depending on cell line, assay and agent used. In order to avoid false indications of toxicity and obtain reliable results, multiple assays should ideally be used (Fotakis and Timbrell, 2006). If not for the time constraints on the study, a neutral red retention assay measuring lysosomal function would have been carried out alongside the MTT assay.

Conflicting results have been reported throughout the literature concerning the affect TiO<sub>2</sub> nanoparticles can have on cell viability, with some studies obtaining positive results (Acar *et al.*, 2015; Coccini *et al.*, 2015; Gosch *et al.*, 2010; Wang *et al.*, 2007) and some reporting negative results (Petkovic *et al.*, 2011; Valdiglesias *et al.*, 2013; Wagner *et al.*, 2009; Zhang and Sun, 2004). The variability in results between studies may be due to experimental differences. Differences in experimental conditions such as temperature of laboratory, spectrophotometer sensitivity and batch of culture medium have been shown to influence the results of nanoparticles with intermediate levels of toxicity, such as TiO<sub>2</sub>, causing varied results between laboratories where the same methods and procedures were used (Lanone *et al.*, 2009). The cell line used may also have an effect on levels of toxicity reported. Some cells are much more sensitive than others (Filograna *et al.*, 2015). The crystalline structure, particle size, coating and interaction with the media can influence the surface charge, sedimentation, aggregation and hence toxicity of TiO<sub>2</sub> nanoparticles (Holmberg *et al.*, 2013; Jugan *et al.*, 2012; Li *et al.*, 2004; Ma *et al.*, 2010; Liu *et al.*, 2011; Marquez-Ramirez *et al.*, 2012; Oberdörster *et al.*, 2007; Petkovic *et al.*, 2011).

The presence or absence of fetal bovine serum (FBS) in media can also alter cell viability. A portion of SH-SY5Y cells grown in medium without serum underwent apoptosis and had altered cell morphology, indicating the need for consistency between experimental procedures. FBS also changes the mean size of nanoparticle aggregates, reducing levels of aggregation (Macleod *et al.*, 2001). The TiO<sub>2</sub> nanoparticles used in this study were suspended in water. It has been shown that nanoparticles suspended in water are prone to aggregation due to the hydration and reduction of electrostatic repulsion (Ates *et al.*, 2013; Marquez-Ramirez *et al.*, 2012). Aggregate size increases with concentration causing the degree of dispersion caused by media to become less pronounced (Doak *et al.*, 2009). This may explain with no cytotoxicity was observed at concentrations as high as 3000 $\mu$ M and 10,000 $\mu$ M; despite the use of FBS; aggregates may have been too large to enter the cells readily. Hackenberg *et al.*, (2010) prepared anatase TiO<sub>2</sub> nanoparticle dilutions in the same way as this study, but found that sonication did not prevent aggregation.



Despite the negative results obtained from the MTT assay, positive results were obtained from the alkaline comet assay. As seen in figure 7, cells treated with 3000 $\mu$ M TiO<sub>2</sub> nanoparticles had a % tail DNA significantly higher than that of the control ( $P < 0.05$ ). This increase in % tail DNA is an indicator of DNA damage and can be seen in figure 8. The percentage of DNA in the tail is the amount of DNA that has migrated out of the nucleus and is directly proportional to the amount of DNA damage the agent has caused (Kumaravel and Jha, 2006). Since the alkaline comet assay is not specific for double strand breaks, this DNA damage could be excision repair sites or single strand breaks. To determine the type and extent of genotoxic damage caused by the TiO<sub>2</sub> nanoparticles other assays such as the micronucleus assay and  $\gamma$ -H2AX foci detection assay can be used (Jugan *et al.*, 2012; Trouiller *et al.*, 2009). Other studies both *in vivo* and *in vitro* have also reported TiO<sub>2</sub> nanoparticle induced genotoxic damage (Botelho *et al.*, 2014; Ghosh *et al.*, 2010; Jugan *et al.*, 2012; Petkovic *et al.*, 2011; Shukla *et al.*, 2011; Trouiller *et al.*, 2009; Valdiglesias *et al.*, 2013; Vevers and Jha, 2008) although mostly at concentrations lower than those used in this study. Trouiller *et al.*, (2009) found genotoxic effects using a similar concentration to that used in this study. They found that orally administered TiO<sub>2</sub> nanoparticles at 600 $\mu$ g/ml induced micronuclei, DNA deletions and  $\gamma$ -H2AX foci formation, an indicator of double strand breaks in mice *in vivo*. Similar to his study, Valdiglesias *et al.*, (2013) and Ursini *et al.*, (2014) found that TiO<sub>2</sub> nanoparticles were able to induce DNA damage yet had little effect on cell viability. They found that anatase TiO<sub>2</sub> nanoparticles were able to induce genotoxic damage assessed by the comet assay. These results were however obtained at much lower, environmentally relevant concentrations (80-150 $\mu$ g/ml) indicating the variability of *in vitro* nanoparticle toxicity results. Studies have also reported negative results for TiO<sub>2</sub> induced genotoxicity. Naya *et al.*, (2012) found no increase in % tail DNA after intratracheal instillation in rats at concentrations as high as 5.0mg/kg body weight. Similarly Hackenberg *et al.*, (2011) also found that TiO<sub>2</sub> nanoparticles did not induce genotoxicity assessed by the comet assay, despite reaching the nucleus of lymphocytes and Linnainmaa *et al.*, (1997) found TiO<sub>2</sub> nanoparticles did not increase micronuclei number.

The production of reactive oxygen species (ROS) is often held accountable for the genotoxic damage caused by the TiO<sub>2</sub> nanoparticles due to the high correlation between ROS levels and genotoxicity, and the negative results often obtained by mutation assays, ruling mutation out as a mechanism for genotoxicity (Chen *et al.*, 2014; Jugan *et al.*, 2012; Shukla *et al.*, 2011). TiO<sub>2</sub> has been shown to significantly reduce levels of glutathione, reduce catalase activity, induce mitochondrial common deletion and increase levels of reactive oxygen species, and oxidise pyrimidine and purine bases, indicators of oxidative stress (Jaeger *et al.*, 2012; Jugan *et al.*, 2012; Reeves *et al.*, 2008; Saquib *et al.*, 2012; Sekar *et al.*, 2011; Trouiller *et al.*, 2009).

One proposed mechanism for ROS mediated genotoxicity is the inactivation of NER and BER DNA repair pathways by reactive oxygen species (Chen *et al.*, 2014). Valdiglesias *et al.*, (2013) however found no evidence for ROS formation or oxidative damage despite finding positive results for dose-dependent apoptosis, altered cell cycle and DNA strand breaks suggesting TiO<sub>2</sub> nanoparticles may induce genotoxic damage by means other than ROS. Similarly Demir *et al.*, (2015) found significant TiO<sub>2</sub> nanoparticle induced DNA damage assessed by the comet assay, but negative results from the modified comet assay using FPG enzyme suggesting the DNA damage was not induced by oxidative stress. A modified comet assay or electron



spin resonance could be carried out to determine if the genotoxic damage seen in this study was due to oxidative damage (Reeves *et al.*, 2008). Mitochondrial DNA damage, measured by mitochondrial common deletion could have also been employed in this study as a biomarker of oxidative stress induced genotoxicity, (Jaeger *et al.*, 2012).

Many factors may influence the variability in genotoxic results obtained such as cell lines and media used. Electrostatic interactions are important for DNA adsorption. DNA is a negatively charged polymer and is more likely to adhere to TiO<sub>2</sub> nanoparticles if they are positively charged. The pH of the surrounding media has been shown to influence the charge of TiO<sub>2</sub>, with it becoming more negatively charged at pH >7 and positively charged at pH<6. The concentration of NaCl in media can influence TiO<sub>2</sub> DNA adsorption, and cause variable results (Zhang *et al.*, 2014). Another reason for the varied results obtained for TiO<sub>2</sub> genotoxicity across the literature is particle size and crystalline structure. Smaller nanoparticles have been found to have a higher genotoxic potential than larger nanoparticles and anatase nanoparticles more so than rutile due to their photocatalytic properties (Chen *et al.*, 2014).

Field emission scanning electron microscopy was used to assess the morphology of the cells following 24 and 48 hour exposure to 300µM and 1000µM TiO<sub>2</sub> nanoparticles. No clear morphological differences were observed in any of the cells. This experiment had a low cell count preventing a quantitative analysis. If this experiment were to be repeated, cell counts could be carried out in each treatment condition. These results are similar to Valdiglesias *et al.*, (2013) who also reported no cytotoxic effect or morphological alterations in SH-SY5Y cells exposed to TiO<sub>2</sub>. X-ray microanalysis was used to determine the presence and location of the TiO<sub>2</sub> nanoparticles. As seen in figures 8 and 9, TiO<sub>2</sub> was present in the treated cell samples. The TiO<sub>2</sub> was more commonly found attached to the cells rather than in the surrounding media (data not shown). This is due to weak attractive forces such as Van der Waals forces and capillarity interactions taking place between single and aggregates of TiO<sub>2</sub> and the SH-SY5Y cell membranes. Treated cells show a tendency to increase adhesive properties during exposure, with Van der Waals and electrostatic forces increasing with incubation time (da Rosa, 2013).

Previous studies have shown that neuronal cells are capable of internalising both single and aggregates of TiO<sub>2</sub> nanoparticles in a time dependant manner (Kenzaoui *et al.*, 2012; Huerta-Garcia *et al.*, 2014; Marquez-Ramirez *et al.*, 2012; Valdiglesias *et al.*, 2013). The mechanism of TiO<sub>2</sub> uptake is still unclear although previous studies have suggested mechanisms may include endocytosis, cytoskeleton protein interaction (Marquez-Ramirez *et al.*, 2012), destabilisation of the membrane (Valant *et al.*, 2012) and the binding of biomolecules from culture medium to nanoparticles to form nanomaterial-protein coronas, masking the nanoparticles identity (Bramini *et al.*, 2014; Cedervall *et al.*, 2007; Doak *et al.*, 2009; Kim *et al.*, 2007; Lynch and Dawson, 2008; Michaelis *et al.*, 2006).

It is unclear in this study whether cells were internalised in the images obtained from x-ray microanalysis as they do not reveal whether the TiO<sub>2</sub> nanoparticles are attached to the surface of the cells or inside them. SH-SY5Y cells used in this study grow as a mixture of both floating and adherent cells (ATCC, 2014). Cells may adhere to the surface of the cover slips after exposure to the TiO<sub>2</sub>, trapping TiO<sub>2</sub>

nanoparticles underneath the cells. This can cause problems using simple imaging techniques as it is unclear whether the nanoparticles are inside the cells, attached to the cell membrane or underneath them. More advanced methods such as flow cytometry and transmission electron microscopy could be used to determine internalisation. Such methods would have been used if not for the time and financial restraints on this study.

Floating cells pose a problem for all assays and experiments in this study. Dead cells have been seen to detach from the well surface, particularly cells treated with copper (positive control). This suggests that as a cell becomes more damaged, it is more likely to detach. All media was removed and replaced for all experiments. This may have resulted in the removal of healthy or damaged cells at all concentrations.

*In vitro* cytotoxicity assays can be used to predict human toxicity, providing a cheaper and quicker alternative to *in vivo* tests and reducing the number of animals used in experiments (Fotakis and Timbrell, 2006). The results obtained from this study and other studies must be obtained from environmentally relevant concentrations to evaluate potential risk to human health. It is unclear exactly what concentrations of TiO<sub>2</sub> nanoparticles humans are exposed to on a daily basis, and of this how much accumulates in the brain. *In vivo* mammalian studies in mice have found TiO<sub>2</sub> in the hippocampus in concentrations ranging from 0.13 to 0.3 µg/ml following 500µg exposure (Wang *et al.*, 2008a and Wang *et al.*, 2008b). It has also been estimated that the average 3-4 year old male in the western world may consume 2mg TiO<sub>2</sub>/kg BW per day (Weir *et al.*, 2012). Since little data is available to predict the amount of TiO<sub>2</sub> human brains may be exposed to care has to be taken when determining safety levels. In order to utilise the data from *in vitro* toxicity assays which reflects intrinsic toxic potential, more *in vivo* studies are necessary to determine expressed toxicity which takes into account appropriate route of exposure, metabolism, excretion and DNA repair capabilities of the tissue.

## Conclusion

The vast use of TiO<sub>2</sub> nanoparticles in medical, food and industrial processes causes humans to be exposed to the nanoparticles on a daily basis. Despite *in vivo* and *in vitro* toxicity tests on multiple target organs, systems and cells, there is still a clear lack of understanding regarding the mechanisms and extent of TiO<sub>2</sub> nanoparticle toxicity. In conclusion, the results from this study show that although TiO<sub>2</sub> nanoparticles did not reduce cell viability, they can, at high concentrations, induce significant DNA damage. These results contribute to the knowledge of titanium dioxide nanoparticle impacts on human health. Further research is needed to determine mechanisms of toxicity.

## Acknowledgements

The author would like to thank Professor Awadhesh Jha, for his support, guidance and knowledge as supervisor. The author would also like to thank Rajan Maynard, William Vevers, Lynne Cooper, Andy Atfield and Shannon O'Sullivan for their guidance and assistance within the laboratory. The author would also like to thank Professor Roy Moate, Pete Bond and Glen Harper, for assistance using the scanning electron microscope and for guidance when preparing samples, and finally Dr. Richard Maunder for guidance.

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